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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

The WTH3 gene was obtained by its N-terminal sequence that was isolated by the Methylation Sensitive-Representational Difference Analysis (MS-RDA) technique due to its hypermethylation in the human multidrug resistant (MDR) breast cancer cell line MCF7/AdrR. The WTH3 gene product is 89% and 91% identical to the human Rab6 and Rab6c proteins, but possesses an elongated C-terminal region that contains 46 extra amino acids. Nevertheless, we consider WTH3 a new member of the Rab6 gene family. Semi-Quantitative RT-PCR results showed that WTH3 was 15 and 4 times down regulated in MCF7/AdrR and MES-SA-Dx5 MDR cell lines. Permanent expression of the WTH3 transgene in MDR cell lines increased to varying degrees their sensitivity to several anti-cancer drugs, which include doxorubicin (DOX), taxol, vinblastine, vincristine, and etoposide, as compared to the control sublines transfected with the empty vector. Flow cytometry and fluorescence microscope experiments suggest that the WTH3 transgene stimulates the host's uptake and retention of DOX. These results imply that the WTH3 gene plays a role(s) in MDR phenotype development in breast cancer cells.

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The Annual Report for Award Number DAMD17-00-1-0383

The Proposal Title: Search for New Multidrug Resistant Genes by Methylation Sensitive Representational Difference Analysis (MS-RDA) in Breast Cancer

Introduction

The overall goal of the proposal funded by the Department of Defense Breast Cancer Research Program is to search for genes whose altered expressions are regulated by DNA methylation and are involved in developing the multidrug resistance (MDR) phenotype in human breast cancer cells. Our specific aims are:

1. to apply the Methylation Sensitive-Representational Difference Analysis (MS-RDA) technique to produce Differentially Methylated Genomic Fragments (DMGFs) from the doxorubicin (DOX) induced MDR human breast cancer cell line, MCF7/AdrR, and its parental cell line, MCF7/WT.
2. to define the abnormally methylated chromosome regions where DMGFs are located.
3. to use the DMGFs to discover novel genes whose expression, or enhanced expression, and silencing, or reduced expression, are related to the MDR phenotype.
4. to study the biological functions of candidate genes, and the roles they play in developing the MDR phenotype.

In the following paragraphs a detailed account of our achievements will be presented.

Body

During the first two years, MS-RDA, a modern technology described in the grant application, was successfully employed to analyze differential DNA methylation patterns in MCF7/AdrR versus MCF7/WT cells. This study led to the discovery of several novel genes including *Rab6c* and *WTH3*. The MDR related functions of the *Rab6c* gene have been investigated and published (1). In this report, we will present detailed information regarding the analysis of *WTH3*'s MDR related characteristics that have also been recently published (2).

Materials and Methods

Semi-Quantitative RT-PCR (SQRT-PCR) and RT-PCR.

Total RNAs were isolated from cell lines by the RNA isolation kit, RNA STAT-60 (TEL-TEST, Inc.). The first strand cDNA was synthesized by the SuperScript Preamplification System Kit (Life Technologies, Inc.). SQRT-PCR was performed by utilizing PW3-1, the *WTH3* gene sense primer, and the anti-sense primer, 5'-GCTGCTACACGTCGAAAGAGC-3', while the cDNAs of MCF7/AdrR, MCF7/WT, MES-SA/Dx5, and MES-SA served as templates. The length of the *WTH3* PCR product was 341 bps. The sense and anti-sense primers for *G3PDH* (internal control) were 5'-CGGAGTCAACGGATTTGGTCGTAT-3' and 5'-AGCCTTCTCCATGGTGGTGA AGAC-3'. The length of the *G3PDH* PCR product was 284 bps. To confirm exogenous *WTH3* gene expression in the stable cell line, PW3-RACE1 (2) was

employed as the sense primer. A sequence in the pcDNA3.1 (Invitrogen) vector polylinker, 5'-CACTGTGCTGGATATCTGCAG-3' (PV-2) (2), was used as anti-sense primer to synthesize the *WTH3* transgene fragment (226 bps). The sense and anti-sense primers for generating the *β-actin* fragment (495 bps), which served as a control, were 5'-GACGACATGGAAGATCTGG-3' and 5'-ATCGGGCAGCTCGTAGCTCTTCC-3'. PCR and quantification of PCR products were performed as described (1).

Generation of stable cell lines

PCR generated *WTH3* was subcloned into a mammalian expression vector, pcDNA3.1 (Invitrogen), to create pcDNA3.1/WTH3. The insert sequence was confirmed by DNA sequencing. MCF7/AdrR and MES-SA/Dx5 cells grown to 60% confluence in 60 mm dishes were transfected with 2 μg of either pcDNA3.1 (control), or the pcDNA3.1/WTH3 plasmid, using calcium phosphate precipitation (5 Prime 3 Prime Inc). The transfected cells were maintained in medium supplied with 200 and 250 μg/ml of neomycin analogue G418 (GIBCO), respectively. Stable G418 resistant populations of G418 resistant MCF7/AdrV (vector alone) and MCF7/Adr6c (Rab6c) cells were acquired after two weeks of selection. The individual clones were obtained by limiting dilution.

Cell growth inhibition

Approximately 1 X 10^3 per/well of WTH3 or the empty vector transfectants were seeded in a 96 well plate (Corning Costar) and grown overnight. DOX, cis-platin (CISP), etoposide (VP16), taxol (TAX), vinblastine (VBL), and vincristine (VCR) anti-cancer drugs (Sigma) were serially diluted into ten concentration. Each group of four wells received one drug concentrations. After a 6 day incubation period, the cells were treated with 3-[4,5-Dimethylthiazol-Z-yl]-2-5-diphenyl-tetrazolium bromide (MTT), which stains living cells. IC50 was quantitatively evaluated at 595 nm by a software, EZ-ED50 (1.11 Version, Perrella Scientific, Inc.), in a microplate reader (3550, BIO-RAD).

Flow Cytometry.

Approximately 5 x 10^5/well of *WTH3* or the empty vector transfected cells were seeded in 6 well dishes. Since the intracellular fluorescence intensity was tested at seven time points, seven wells for each transfectant group were prepared. One well for each group was not treated by DOX and served as the 0 time control. Each group of six wells was incubated with 30 μg/ml of DOX in 1 ml of culture media for 2 hrs at 37°C in 5% CO_2. To measure the intracellular fluorescence intensity, the cells were washed twice in ice cold PBS and trypsinized. Cells were centrifuged at 1000 rpm for 5 min to pellet and then re-suspended in 300 μl of 1% paraformaldehyde. The intracellular DOX fluorescence content was analyzed by flow cytometry as described (33). The fluorescence intensity was measured at 1 and 2 hour intervals during the DOX incubation period. The remaining four wells of each transfectant were then washed twice with ice cold PBS and incubated in a DOX free culture medium for 4 hours. The intensity of fluorescence at 1, 2, 3, and 4 hours of chase was measured.

Fluorescence Microscopic Assay.

Approximately 5 x 10^4/well of *WTH3* or the empty vector transfectants were cultured in 6 well dishes. Individual wells were incubated with 10 μg/ml of DOX in media for two hours. Media was then removed and the cells were washed with ice cold PBS, followed by the addition of fresh media without DOX. Cells were immediately examined for fluorescence at 488 nm excitation wavelength by fluorescence microscopy.

Results

The WTH3 Gene was Under-Expressed in MCF7/AdrR and MES-SA/Dx5 Cells.

Since the N-terminal portion of the *WTH3* gene, *W3*, was hypermethylated in MCF7/AdrR cells, the *WTH3* gene's expression level in MCF7/AdrR and MCF7/WT cells was analyzed by SQRT-PCR using gene specific primers. The predicted size of the *WTH3* PCR product was 341 bps, while 284 bps was the anticipated length of the *G3PDH* gene, which served as a quantitative control. The results showed that the expression of *WTH3* in MCF7/AdrR cells was 15 times less than that in MCF7/WT cells (see Fig. 3. in Ref. 2). In addition, the *WTH3* gene's expression level was evaluated in the MDR cell line MES-SA/Dx5 and its non-MDR counterpart MES-SA. We found that *WTH3* was 4 times less expressed in MES-SA/Dx5 as compared to MES-SA cells (see Fig. 3. in Ref. 2).

Drug Induced WTH3 Transfectants Growth Inhibition Assays.

Hypermethylation and low expression of the *WTH3* gene in MCF7/AdrR and MES-SA/Dx5 cells indicated that this gene might be a negative regulator for drug resistance. To test this hypothesis and obtain reliable results, both MCF7/AdrR and MES-SA/Dx5 were transfected with the *WTH3* gene to generate stable cell lines. Another reason for using the MES-SA/Dx5 line was that it exhibited a much weaker MDR phenotype than MCF7/AdrR. Therefore, the *WTH3* gene could have a stronger influence on MES-SA/Dx5 as compared to MCF7/AdrR cells.

The *WTH3* gene was generated by PCR and subcloned into the pcDNA3.1 vector to create pcDNA3.1/WTH3. This construction and the vector were separately introduced into the host cells by calcium phosphate precipitation procedure. The transfected cells were maintained in medium containing G418 for selecting stable transformed populations. After verifying, by measuring their IC50s, that the stably populations harboring the transgene exhibited higher sensitivity to DOX than the controls integrated with the empty vector, limiting dilution was carried out to obtain stable cell clones. Three MCF7/AdrR and five MES-SA/Dx5 individual transfectants were selected. Limiting dilution procedures were also carried out to obtain five individual MCF7/AdrR or MES-SA/Dx5 transfectants integrated with the empty vectors (negative controls). The expression of the exogenous *WTH3* gene in each clone was confirmed by RT-PCR, where the gene specific primer PW3-1 and pcDNA3.1 poly-linker primer PV-2 were used. All clones, along with their controls, were utilized for drug induced cell growth inhibition assays. IC50s for the anti-cancer drugs DOX, CISP, TAX, VBL, VCR, and VP-16 were evaluated. We found that the transgene increased the MCF7/AdrR clones' sensitivity to DOX, TAX, VBL, and VCR by factors ranging from 2 to 6 fold (data not shown). However, the

transgene had a much stronger influence on four out of five MES-SA/Dx5 clones. For example, both clone #2 (ME-2) and #8 (ME-8) had significant increased sensitivity to DOX, TAX, VBL, VCR, and VP-16 as compared to the five controls containing the empty vector (ME-V). The sensitivity of clones #4 (ME-4) and #7 (ME-7) to the same drugs was also elevated by the transgene but responded to a lesser extent. Clone #5, which expressed a very low level of the transgene, exhibited a drug resistant phenotype similar to that of all five control sublines (data not shown). In the next paragraph, detailed information on the IC50 measurements of clones ME-2, ME-4, and ME-7 is presented.

To determine the IC50s; ME-2, ME-4, ME-7, and five ME-V sublines were kept in media without the drug, or with the other ten drug concentrations for 6 days. More than three individual experiments were performed for each cloned cell line. The IC50 measurements found that the *WTH3* transgene increased the host clone's sensitivity to DOX, TAX, VBL, VCR, and VP-16, but not to CISP, to varying degrees as compared to the control cell lines which exhibited the original MDR phenotype (see Table 1. in Ref. 2. Since all five ME-V controls had similar resistance to the drugs tested, only the results obtained from one ME-V were listed.). The sensitivity of the ME-2 clone to DOX, TAX, VBL, VCR, and VP-16 was 35, 216, 45, 174, and 26 times greater than that of the control cells (see Table 1 and Fig. 4A. in Ref. 2). The ME-4 clone's sensitivity to the same anti-cancer drugs was 8, 22, 8, 13, and 4 times higher than the control cells, while that of the ME-7 clone was elevated 14, 30, 16, 28, and 8.5 times relative to the control (see Table 1. in Ref. 2). Expression of the *WTH3* transgene in ME-2, ME-4, and ME-7 was verified by RT-PCR (see Fig. 4B. in Ref.2). Densitometer analysis found that the expression level of the transgene in ME-2 was 1.6 and 2.2 times higher than that in ME-7 and ME-4, which could be a reason for ME-2 exhibiting higher drug sensitivity.

Flow Cytometry Assay for DOX Uptake and Retention in ME-2 Cells.

DOX is fluorescent, and this attribute provides easy monitoring of its intracellular accumulation by flow cytometry. Thus, ME-V and ME-2 cells were incubated with DOX for two hours, after which the cells were washed and remained in a medium without DOX for four hours. DOX uptake and retention in the cells was quantitatively determined at different time points (see Material and Methods). The ME-V cells displayed no significant increase in cellular fluorescence after DOX incubation. However, the ME-2 cells, which contained the *WTH3* transgene, displayed greatly increased DOX uptake (see Fig. 5A. in Ref.2). Furthermore, the intensity of fluorescence in ME-2 and ME-V cells was measured at four time points after the cells were washed with PBS buffer. The results showed that the fluorescence remained much stronger, even after 4 hours of chase, in ME-2 than that in ME-V cells (see Fig. 5B. in ref.2). These findings clearly demonstrated the positive effect of the *WTH3* gene on DOX uptake and retention in the host cells.

Fluorescence Microscopy of DOX Accumulation in ME-2 Cells.

Flow cytometry experiments found that the *WTH3* gene stimulated the host cells' uptake and retention of DOX. To visually determine the intercellular location of the accumulated DOX, fluorescence microscopy was performed. ME-V and ME-2 cells were treated with DOX for 2 hrs, after which the drug was washed away. The fluorescence accumulation and distribution in

the control and test cells were examined under a microscope (see Fig. 6A-D. in Ref. 2). The control cells with the drug resistant phenotype only contained trace amounts of fluorescence (see Fig. 6B. in Ref. 2), while strong fluorescence was displayed in the nucleus and cytoplasm of the ME-2 cells (see Fig. 6D. in Ref. 2). The intensity of fluorescence continued to remain strong even after 3 hours of chase in the ME-2 cells (data not shown).

Key Research Accomplishments

In the past year our studies have included the following key accomplishments: 1).). The full length *WTH3* cDNA has been uncovered and its expression levels in MDR MCF7/AdrR and its non-MDR counterpart MCF7/WT cells were evaluated by RT-PCR.

Reportable Outcomes

1. Stable cell lines integrated with the *WTH3* gene have been established.
2. The *WTH3* gene sequence has been submitted to the NIH GenBank, and its accession number can be found in Reference 2.
3. A research opportunity has been applied by this funding to Dr. J. Shan.

Conclusions

WTH3 encodes 254 amino acids with 22 and 19 substitutions as compared to Rab6 and Rab6c. These substitutions are evenly distributed, and this would indicate that *WTH3* is not an alternative splice product of *Rab6*. Four domains of the K-ras protein involved in GTP/GDP binding are also included in WTH3 (corresponding to residues 20-27, 67-73, 123-130, and 153-159) (3). This implies that *WTH3* encodes a small G protein. However, differing from Rab6 and Rab6c, WTH3 does not possess any cysteine near its COOH terminus. The existence of cysteine is assumed to be necessary for many G proteins' fatty acylation, membrane association, and biological function (4). The C-terminal diversification suggests that the *WTH3* protein might have its own unique utilities.

We demonstrated that the *WTH3* gene was less expressed in MCF7/AdrR and MES-SA/Dx5 cells than in their parental cell lines. Increased expression of the *WTH3* gene in both MDR cell lines converted their MDR phenotype. In the past, we reported that the *Rab6c* gene was involved in MDR in MCF7/AdrR cells. Thus, the *Rab6* genes play roles involved in MDR in breast cancer cells. However, differing from known MDR genes, which function as positive regulators for MDR development, WTH3 and Rab6c proteins function as negative regulators. It will be interesting to understand the biological pathway(s) of each member in the *Rab6* gene family and explore the possible relationships between them and P-glycoproteins.

Our studies provide evidence that in addition to known drug resistant genes, *MDR1*, glutathione transferase and glutathione peroxidase (positive regulators), a small G protein encoded by *WTH3*, also participates in drug resistant phenotype development in MCF7/AdrR

cells. This finding did not surprise us considering *Rab6* and *WTH3*'s structural similarities, and the fact that Rab6 is associated with Golgi and trans-Golgi network membranes (Martinez et al., 1994; Mayer et al., 1996), and acts as an inhibitor in anterograde transport within this organelle (Martinez et al., 1994; Martinez et al., 1997; Martinez and Goud, 1998). This feature makes one contemplate whether *Rab6* genes inhibit anterograde transport, or stimulate retrograde transport of a drug. The data obtained from this proposal suggest that multiple biological processes are involved in the evolution of MDR in cancer cells. The search for those unknown elements, and the study of their biological functions, will be of great help in understanding the mechanisms that cause clinical multidrug resistance.

The results presented in this report demonstrate that the pace of our research went along well with the schedule planned in the Statement of Work. We are confident that the original grant proposal is scientifically sound and our goal will be reached.

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Appendices

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Rab6c, a new member of the *Rab* gene family, is involved in drug resistance in MCF7/AdrR cells

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Abstract

A new *Rab6* homolog cDNA, *Rab6c*, was discovered by a hypermethylated DNA fragment probe that was isolated from a human multidrug resistant (MDR) breast cancer cell line, MCF7/AdrR, by the methylation sensitive-representational difference analysis (MS-RDA) technique. *Rab6c* was found to be under-expressed in MCF7/AdrR and MES-SA/Dx5 (a human MDR uterine sarcoma cell line) compared with their non-MDR parental cell lines. MCF7/AdrR cells expressing the exogenous *Rab6c* exhibited less resistance to several anti-cancer drugs, such as doxorubicin (DOX), taxol, vinblastine, and vincristine, than the control cells containing the empty vector. Flow cytometry experiments confirmed that the transfectants' diminished resistance to DOX was caused by increased drug accumulation induced by the exogenous *Rab6c*. These results indicate that *Rab6c* is involved in drug resistance in MCF7/AdrR cells.

Keywords: Gene expression; Gene transfection; G protein; IC50; MS-RDA

1. Introduction

Drug resistance to a broad spectrum of chemotherapeutic agents is a major obstacle in the clinical treatment of human cancer (Juliano and Ling, 1976; Goldie and Coldman, 1983; Roninson et al., 1984). In the last decade, several genes involved in multi drug resistance (MDR) have been identified. The most extensively studied of these genes are *MDR1*, which encodes a P-glycoprotein (P-gp), and *MRP*, which encodes an MDR-associated protein (Chen et al., 1986; Gros et al., 1986a,b; Cole et al., 1992). Both genes' function is to reduce intracellular drug concentration either directly, by acting as a drug pump, or indirectly, through other mediators (Zaman et al., 1994; Bolhuis et al., 1997). However, over-expression of MDR1/MRP does not completely explain the drug resistance phenomenon. Clearly, other unknown drug resistant mechanisms are involved (Nooter and Stoter, 1996; Kaye, 1998). Therefore, searching for those mechanisms, with the help of modern techniques, may be of great importance in increasing the efficiency of cancer therapy.

Since DNA methylation plays an important role in the regulation of gene expression in mammals (Adams and Burdon, 1982; Doerfler, 1983; Riggs and Jones, 1983; Graff et al., 1995; Kass et al., 1997), we employed methylation-sensitive representational difference analysis (MS-RDA) (Ushijima et al., 1997; Yuan et al., 1999), which is an RDA-based technology (Lisitsyn et al., 1993), to analyze differential DNA methylation in MCF7/AdrR and its parental non-MDR cell line, MCF7/WT, with the hope of discovering new MDR-related genes. Both cell lines were gifts from Dr K. Cowan, of the National Cancer Institute (Batist et al.,

Abbreviations: aa, amino acid(s); bp, base pair(s); cDNA, DNA complementary to RNA; MDR, multidrug resistance; MS-RDA, methylation-sensitive representational difference analysis; MTT, 3-[4,5-dimethylthiazol-Z-yl]-2-5-diphenyl-tetrazolium bromide.

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1986). The MCF7/AdrR cells were generated from MCF7/WT cells by gradually increasing doxorubicin (DOX) concentration in the culture media. These cells with increased activity of glutathione transferase and glutathione peroxidase (Batist et al., 1986), as well as high-level expression of the *MDR1* gene (our observation, data not shown) were found to exhibit cross-resistance to a wide range of anti-cancer drugs. MS-RDA generated several DNA fragments that were hypermethylated in MCF7/AdrR cells. One of them, *W3*, which was found to be homologous to the human *Rab6* gene, was used as a probe to search for a putative full-length coding fragment from human cDNA libraries. As a result, a new member of the *Rab* gene family, *Rab6c*, was obtained. In the *Rab6c* sequence there are 31 bp substitutions clustered in a short 96 bp region relative to *Rab6*. This indicates that *Rab6c* could be a spliced variant of the *Rab6* gene. A similar hypothesis was also suggested by the Opdam group, who directly submitted a cDNA sequence, which is identical to *Rab6c*, to the NIH GeneBank. Quantitative RT-PCR (QRT-PCR) found that *Rab6c* was under-expressed in MCF7/AdrR, and MES-SA/Dx5 whose MDR phenotype was induced from the parental cell line MES-SA by DOX (ATCC Inc.) (Harker et al., 1983; Harker and Sikic, 1985). The cell growth inhibition assays discovered that the MCF7/AdrR cells expressing the exogenous *Rab6c* increased sensitivity to DOX, taxol (TAX), vinblastine (VBL), and vincristine (VCR), but not to cisplatin (CISP) and etoposide (VP-16). The flow cytometry assay results confirmed that *Rab6c* increased DOX retention in the host cells. These findings indicate that *Rab6c* could play a role in the development of drug resistance in MCF7/AdrR cells.

2. Materials and methods

2.1. Cell lines

MCF7/AdrR, MCF7/WT, MES-SA/Dx5, and MES-SA cells were grown under the same conditions [RPMI medium 1640 (GIBCO), supplemented with 10% fetal bovine serum (FBS) (GIBCO), penicillin (100 μg/ml), streptomycin (0.25 μg/ml), and glutamine to a final concentration of 2 mM] with the exception of supplying 1 μg/ml and 0.5 μg/ml of DOX (Sigma) to MCF7/AdrR and MES-SA/Dx5 cells.

2.2. MS-RDA

Both MCF7/AdrR and MCF7/WT cell lines were propagated to the logarithmic phase, and the DNAs were isolated by the phenol extraction method. They were then cleaved by the *Hpa*II enzyme. To study hypermethylation events, MCF7/WT DNA was used as tester and MCF7/AdrR DNA was used as driver. MS-RDA was performed as described (Lisitsyn et al., 1993; Yuan et al., 1999).

2.3. DNA sequencing

Plasmids containing the candidate sequence, or PCR products, were sequenced using Dye-Labeled Sequence Kit (Perkin–Elmer) under conditions described by the manufacturer.

2.4. cDNA cloning

A normal human kidney 5′-stretch λgt10 cDNA library, a gift from Dr M. Hamaguchi of Cold Spring Harbor Laboratory, was screened using the *W3* probe. 2×10^5 plaques evenly distributed on 20 plates (150 mm × 15 mm) were transferred onto Hybond N^- membranes. The treatment of the membranes, the preparation of the probes, and the Southern analysis were performed as previously described (Yuan et al., 1999). The phage DNA, with human cDNA inserts, was purified by the λQuick! Spin Kit (BIO101 Inc.) following the instructions of the manufacturer. The individual inserts were released by restriction enzyme *Eco*RI cleavage from the phage DNA arms, then subcloned into the pUC118 plasmid for sequencing.

2.5. RT-PCR and QRT-PCR

Total RNAs were isolated from corresponding cell lines by RNA isolation kit, RNA STAT-60 (TEL-TEST, Inc). The first strand cDNA was synthesized by SuperScript Preamplification System Kit (Life Technologies, Inc.). The sense and anti-sense primers for *Rab6c* and *W3* were 5′-GCTGAGGAAATTCA-AGCTGG-3′ and 5′-CTACTACAGCTGCAGCAG-AATC-3′. *β-actin* internal control sense and anti-sense primers were 5′-GACGACATGGAGAAGATCTGG-3′ and 5′-ATCGGGCAGCTCGTAGCTCTTCC-3′. To confirm the exogenous *Rab6c* expression in the stable cell line, 5′-GATCGAACAATCAGGCTTCAG-3′ was used as sense primer for pcDNA/R6c. A sequence in the polylinker of the pcDNA1.1/Amp vector, 5′-CAGTGTGATGGATATCTGCAG-3′, was used as anti-sense primer to synthesize the transfected *Rab6c* gene fragment. The sense and anti-sense primers for the *β-actin* control were 5′-GACGACATGGAGAAG-ATCTGG-3′ and 5′-TGTAGAGGTAGTCAGTC-AGG-3′. The PCR reaction mixtures included cDNA derived from 250 ng of total RNA, 5 pmol of sense and anti-sense primers for both the *β-actin* and gene to be tested, 200 μM of four deoxynucleotide triphosphate, and 0.25 units of Taq DNA polymerase with reaction buffer (Perkin–Elmer) in a final volume of 50 μl. The target and *β-actin* sequences were amplified in the same

tube. Each cycle of PCR included 30 s of denaturation at 95°C, 60 s of annealing at 59°C, and 60 s of extension at 72°C. For the QRT-PCR, 38 cycles of PCR were carried out, and the amplifications of both the *β-actin* and tested gene were found to be linear over 25 cycles. The PCR products were separated on 2% agarose gels and a quantity for the gene tested was measured by a densitometer (Gel Doc 1000, BIO-RAD) in comparison with the *β-actin* PCR product.

2.6. Generation of stable cell lines

The PCR-generated *Rab6c* was subcloned into a mammalian expression vector, pcDNA1.1/Amp (Invitrogen), to create pcDNA/R6c. The insert sequence was confirmed by DNA sequencing. MCF7/AdrR cells grown to 75% confluence in 60 mm dishes were co-transfected with 20 μg of non-selectable pcDNA1.1/Amp (control), or pcDNA/R6c plasmids and 5 μg of a neomycin expression plasmid using a calcium phosphate precipitation kit (5 Prime 3 Prime Inc). The transfected cells were maintained in medium supplied with 200 μg/ml of neomycin analogue G418 (GIBCO). Stable populations of G418-resistant MCF7/AdrV (vector alone) and MCF7/Adr6c (Rab6c) cells were acquired after 2 weeks of selection, and individual clones were obtained by limiting dilution.

2.7. Cell growth inhibition

Approximately 5×10^3 MCF7/AdrV, or MCF7/Adr6c cells per well were seeded in a 96-well plate (Corning Costar) and grown overnight. The cells were then treated with DOX, CISP, VP-16, TAX, VBL, and VCR anti-cancer drugs (Sigma). Each group of four wells was treated by one of ten different serially diluted drug concentrations. 3-[4,5-Dimethylthiazol-*Z*-yl]-2-5-diphenyl-tetrazolium bromide (MTT), which stains living cells, was introduced after 3 days (stable cell population), or 6 days (stable cell clones) incubation. IC50 was quantitatively evaluated at 595 nm by a program software, EZ-ED50 (1.11 Version, Perrella Scientific, Inc.), in a microplate reader (3550, BIO-RAD).

2.8. Flow cytometry

Approximately 5×10^5 MCF7/AdrR, MCF7/AdrV, or MCF7/Adr6c cells per well, in six-well dishes, were loaded with 30 μg/ml DOX in 1 ml of culture media for 2 h at 37°C in 5% CO_2. Media were then removed and the wells washed with ice-cold PBS, followed by the addition of fresh media without DOX (chase). The cells that were not treated with the drug were used as the control. At various time points (0, 3, and 5 h chase), the media were removed and the cells washed twice in PBS and trypsinized. Cells were centrifuged at 1000*g*, 5 min to pellet and re-suspended in 300 μl of 1% paraformaldehyde. The intracellular DOX content was analyzed by flow cytometry as described (Maslak et al., 1994).

The GeneBank accession numbers for *Rab6c* and *W3* are AF119836 and AF124200.

3. Results

3.1. MS-RDA

The DNA methylation status of MCF7/AdrR cells (driver), and MCF7/WT cells (tester), was analyzed by MS-RDA. To identify the hypermethylated DNA fragments, Southern analysis was performed on both cell lines' genomic DNAs, which were digested by *Hpa*II or the *Msp*I enzyme. *Msp*I is an isoschizomer of *Hpa*II, but not sensitive to C^mCGG. Therefore, *Msp*I could cleave a methylated site that could not be cut by *Hpa*II. Southern analysis results indicated that a fragment, *W3*, was hypermethylated in the MCF7/AdrR DNA because *Msp*I digestion released a band that was not cleaved by the *Hpa*II enzyme (Fig. 1). Sequence analysis of *W3* (Fig. 2A) found that it can be translated into an uninterrupted polypeptide and shared 89% identity with a major portion of a known human gene, *Rab6* (Zahraoui et al., 1989). It appeared that *W3* encompassed a coding region. Therefore, we used *W3* as a probe to search for the full-length gene of interest.

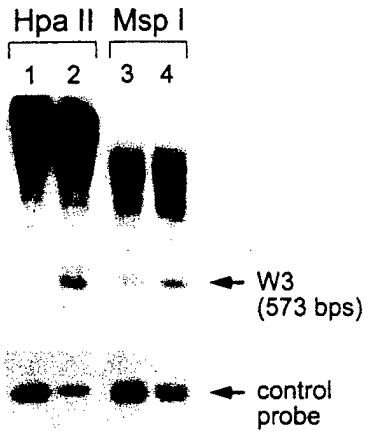

Fig. 1. The results of genomic Southern blot for *W3*. The arrows indicate the fragments that are hybridized with the radiation-labeled *W3* and control probes. In lanes 1 and 2, the *W3* probe hybridized with 6 μg of *Hpa*II-digested genomic DNA isolated from MCF7/AdrR and MCF7/WT cells. In lanes 3 and 4, the probe hybridized with 6 μg of *Msp*I-digested genomic DNA also isolated from MCF7/AdrR and MCF7/WT. The completion of enzymatic digestion for all genomic DNAs was confirmed by a control probe, which was a background DNA fragment isolated by MS-RDA.

A

```
  1 ccgggaggtctctgggctgaggcggcgacagctcctctagttccaccatgtccgcgggcg
 61 gagacttcgggaatccgctgaggaaattcaagctggtgttcctgggggagcaaagcgttg
121 caaagacatctttgatcaccagattcaggtatgacagttttgacaacacctatcaggcaa
181 taattggcattgacttttttatcaaaaactatgtacttggaggatggaacaatcgggcttc
241 ggctgtgggatacggcgggtcaggaacgtctccgtagcctcattcccaggtacatccgtg
301 attctgctgcagctgtagtagtttacgatatcacaaatgttaactcattccagcaaacta
361 caaagtggattgatgatgtcagaacagaaagaggaagtgatgttatcatcacgctagtag
421 gaaatagaacagatcttgctgacaagaggcaagtgtcagttgaggagggagagaggaaag
481 ccaaagggctgaatgttacgtttattgaaactagggcaaaaactggatacaatgtaaagc
541 agctctttcgacgtgtagcagcagctttgccgg
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B

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  1 cagctggctggagcagcatcggtccgggaggtctctaggctgaggcggcggccgctcctc

 61 tagttccacaatgtccacgggcggagacttcgggaatccgctgaggaaattcaagctggt
              M  S  T  G  G  D  F  G  N  P  L  R  K  F  K  L  V

121 gttcctgggggagcaaagcgttggaaagacatctttgatcaccagattcatgtatgacag
      F  L  G  E  Q  S  V  G  K  T  S  L  I  T  R  F  M  Y  D  S

181 ttttgacaacacctatcaggcaacaattggcattgacttttttatcaaaaactatgtactt
      F  D  N  T  Y  Q  A  T  I  G  I  D  F  L  S  K  T  M  Y  L

241 ggaggatcgaacaatcaggcttcagctgtgggatactgcgggtcaggaacgtttccgtag
      E  D  R  T  I  R  L  Q  L  W  D  T  A  G  Q  E  R  F  R  S

301 cctcattcccagttacatccgtgattctgctgcagctgtagtagtttacgatatcacaaa
      L  I  P  S  Y  I  R  D  S  A  A  A  V  V  V  Y  D  I  T  N

361 tgttaactcattccagcaaactacaaagtggattgatgatgtcagaacagaaagaggaag
      V  N  S  F  Q  Q  T  T  K  W  I  D  D  V  R  T  E  R  G  S

421 tgatgttatcatcatgctagtaggaaataaaacagatcttgctgacaagaggcaagtgtc
      D  V  I  I  M  L  V  G  N  K  T  D  L  A  D  K  R  Q  V  S

481 aattgaggagggagagaggaaagccaaagagctgaatgttatgtttattgaaactagtgc
      I  E  E  G  E  R  K  A  K  E  L  N  V  M  F  I  E  T  S  A

541 aaaagctggatacaatgtaaagcagctctttcgacgtgtagcagcagctttgccgggaat
      K  A  G  Y  N  V  K  Q  L  F  R  R  V  A  A  A  L  P  G  M

601 ggaaagcacacaggacagaagcagagaagatatgattgacataaaactggaaaagcctca
      E  S  T  Q  D  R  S  R  E  D  M  I  D  I  K  L  E  K  P  Q

661 ggagcaaccagtcagtgaaggaggctgttcctgctaatctcccatgtcatcttcaaccttt
      E  Q  P  V  S  E  G  G  C  S  C

721  cttcagaagctcactgcttt
```

Fig. 2. (**A**) Nucleotide sequence of *W3*. Nucleotides that are different from *Rab6c* are displayed in bold type. (**B**) Nucleotide and amino acid sequences of the *Rab6c* cDNA and its product. Nucleotides and amino acids that are different from the *Rab6* gene and its product are displayed in bold type.

3.2. Isolation of Rab6c by cDNA library screening

To chose the most suitable cDNA library, two human tissue mRNA panels, MTN™ and MTN™ II (Clontech Inc.), which encompassed 16 different tissues, were hybridized by *W3*. We found that the gene we were searching for was universally expressed (data not shown). Thus, a normal human kidney 5′-stretch λgt10 cDNA library was screened by *W3*. Three individual clones, #4, #5, and #9, approximately 1 kbp, 2 kbp, and 1.2 kbp in length, were discovered. Their phage DNAs were isolated, and cDNA inserts released by *Eco*RI cleavage were subcloned into a pUC118 plasmid for DNA sequencing.

Sequence analysis and homolog searches of the NIH GeneBank found that clones #4 and #9 encoded the same gene, which was 94% identical to the human *Rab6* gene, and 98% identical to its protein product. As a result, we named this cDNA fragment *Rab6c*. Furthermore, the *Rab6c* sequence was 96% homologous, but not identical, to the *W3* probe. In the *W3* sequence, there were 21 base pairs, which were evenly distributed, but did not truncate the putative protein, that differed from those in the *Rab6c* sequence (Fig. 2A). Clone #5 was found to be 100% identical to the human *Rab6* gene. Analogous to the Rab6 protein, the Rab6c protein also contained the same number (208) of amino acids. However, in the middle of *Rab6c*, there is a region containing 96 bp that deviates from the *Rab6* gene by 31 bp variations (Fig. 2B). Nonetheless, this disparity only resulted in three amino acid variations. Since we failed to obtain the putative full-length *W3* gene by screening another cDNA library (human erythro-leukemia 5′-stretch cDNA library, Clontech Inc.), and considering the molecular similarity between *Rab6c* and *W3*, our curiosity led us to explore the possible involvement of *Rab6c* in drug resistance. Our first experiment involved evaluating expression levels of *Rab6c* in MCF7/AdrR versus MCF7/WT, and MES-SA/Dx5 versus MES-SA cells.

3.3. *Rab6c was under-expressed in MCF7/AdrR and MES-SA/Dx5 cells*

To measure *Rab6c* expression levels, QRT-PCR was carried out using paired primers and cDNAs prepared from MCF7/AdrR and MCF7/WT cell lines. Since the nucleotide substitutions in *Rab6c* and *W3* were evenly distributed, designation of primers specific to *Rab6c* was difficult. As a result, we used primers that can amplify both *Rab6c* and *W3*. However, the sense, but not the anti-sense primer, was identical to the sequence in *Rab6*. Therefore, the primers cannot generate a PCR product when *Rab6* is used as a template. The predicted size for the *Rab6c/W3* PCR product was 244 bp, whereas 495 bp was the anticipated length for the *β-actin* gene that served as a quantitative control. The QRT-PCR results demonstrated that the PCR product was seven times less expressed in MCF7/AdrR cells compared with MCF7/WT cells (Fig. 3A). Since the products could be a mixture of *Rab6c* and *W3* sequences, the PCR products generated from both cell lines were subcloned into pUC118 for sequence analysis. Ten clones from each PCR product were analyzed. The results showed that they were *Rab6c* (data not shown). This result was not surprising, since it was apparently consistent with the experience we encountered when we failed to obtain a putative corresponding cDNA from screening two human cDNA libraries using *W3* as the probe. Thus, we verified that *Rab6c* was approximately seven times

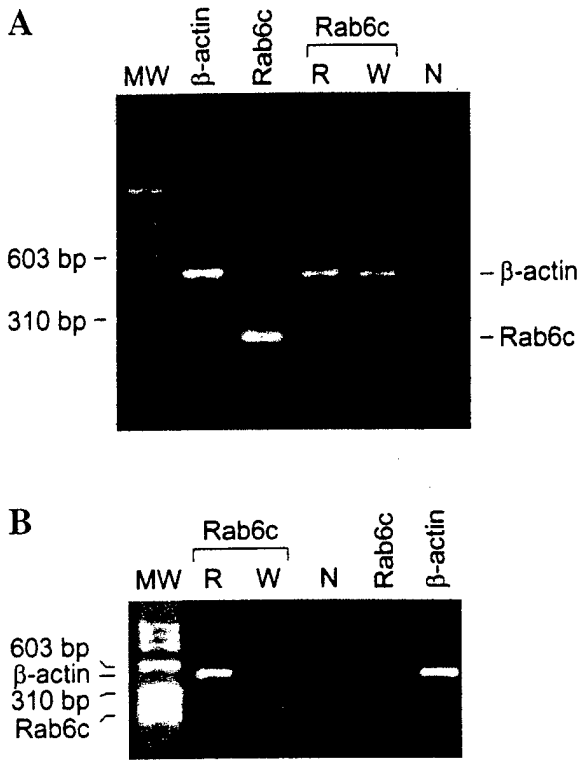

Fig. 3. The results of *Rab6c*-specific QRT-PCR. In (**A**) and (**B**), lane MW represents *Hae*III ϕ174 markers. The *β-actin* and *Rab6c* lanes served as positive controls and were generated using cDNA prepared from MCF7/WT (**A**) and MES-SA cells (**B**). Lanes R and W, under the Rab6c brackets, contain simultaneously generated *Rab6c* and *β-actin* PCR products using cDNA from MCF7/AdrR and MCF7/WT cells (**A**), or from MES-SA/Dx5 and MES-SA cells (**B**). Lane N is the PCR negative control.

less expressed in MDR cells compared with non-MDR cells. To understand whether *Rab6c* expression levels in other MDR cell lines was also changed, QRT-PCR was performed using cDNA prepared from MES-SA/Dx5 and MES-SA. Sequence analysis also verified that the PCR products were *Rab6c*. The results showed that *Rab6c* was 3.5 times less expressed in MES-SA/Dx5 than in MES-SA cells (Fig. 3B).

3.4. *Rab6c transfectants growth inhibition assays*

To find out whether the *Rab6c* cDNA could convert the drug resistance phenotype of MCF7/AdrR cells, stable cell lines would have to be established. Therefore, MCF7/AdrR cells stably harboring expression plasmids for *Rab6c* (MCF7/Adr6c) and pcDNA1.1 empty vectors (MCF7/AdrV), which served as a control, were generated by co-transfection using an expression plasmid for neomycin resistance and selection in G418. The expression of the exogenous *Rab6c* in the MCF7/Adr6c cell population was confirmed by RT-PCR using primers that were based on *Rab6c* and the vector's polylinker sequences. A 488 bp PCR product was amplified from *Rab6c* transfec-

tants, but none appeared in the control cells (Fig. 4A). Before performing limiting dilution to obtain individual transfectants, a time-consuming protocol, we carried out growth inhibition assays on the MCF7/Adr6c and MCF7/AdrV cell populations to estimate the drug-resistance-related potential for *Rab6c*. For this purpose, the IC50 of those cells to an anti-cancer drug, DOX, was measured (IC50 represents drug concentration that results in 50% cell death). If this experiment proved that the *Rab6c* cDNA was capable of reversing the MDR phenotype in MCF7/AdrR cells, individual cell clones would be obtained by limiting dilution and their IC50s for different drugs would be tested. To evaluate the cell populations' IC50 for DOX, ten dilutions of the drug were used. The cells were kept in media containing either none or the other ten drug concentrations for 72 h. After MTT treatment, their IC50s were measured. Four individual experiments were performed for each cell line. The mean IC50±SD of the MCF7/Adr6c cells was 1.53±0.13 μM, whereas that of the control cell was 4.93±0.30 μM. Based on the IC50s, we determined that the *Rab6c* cells exhibited 3.2 times ($P<0.0001$) more sensitivity to DOX than the control cells (Fig. 4B). Before performing limiting dilution, flow cytometry for DOX retention was carried out to confirm further the drug-resistance-related function of *Rab6c*.

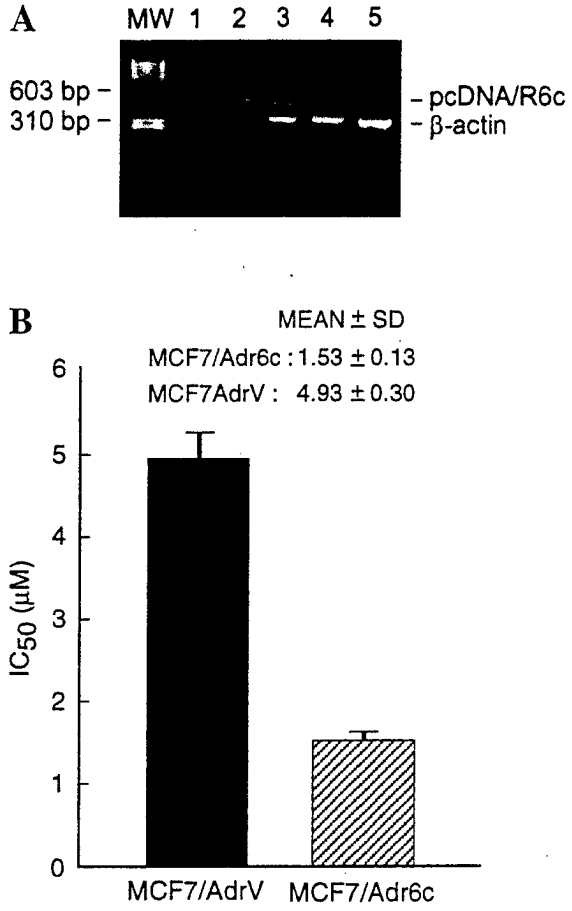

Fig. 4. (A) RT-PCR of the expressed exogenous *Rab6c* in MCF7/Adr6c cells. Lane MW represents *Hae*III ϕ174 markers. Lane 1 is the negative control. Lanes 2 and 5 contain the positive controls for *Rab6c* and *β-actin*. The product in lane 2 was generated by using cDNA prepared from MCF7/Adr6c RNAs and *Rab6c*-specific primers. The product in lane 5 was generated from cDNA prepared from non-transfected MCF7/AdrR cells. Lane 3 contains simultaneously generated *Rab6c* and *β-actin* PCR products by utilizing cDNA that was used in lane 2. Lane 4 only contains *β-actin* generated from cDNAs prepared from MCF7/AdrV cells, although *β-actin*- and *Rab6c*-specific primers were simultaneously added. (B) Growth-inhibition of MCF7/AdrV and MCF7/Adr6c cells caused by DOX. The black and striped vertical columns represent the IC50 of MCF7/AdrV and MCF7/Adr6c cells (mean of four experiments) respectively. The thin bars represent the standard deviation (SD). The differences between the IC50 of MCF7/Adr6c and MCF7/AdrV cells are significant at $P<0.0001$.

3.5. Flow cytometry assay for DOX retention

MCF7/AdrR, MCF7/AdrV, and MCF7/Adr6c cells were analyzed by flow cytometry for retention of DOX in load/chase experiments (Fig. 5). Both parental and MCF7/AdrV cells showed similar increases in fluorescence after loading with the naturally fluorescent DOX, with subsequent loss of fluorescence after 3 h chase and further loss after 5 h chase (Fig. 5B and C). However, after 3 and 5 h of chase, the MCF7/Adr6c cells retained

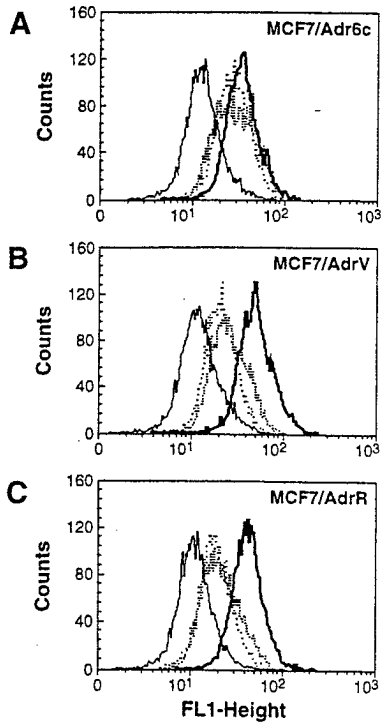

Fig. 5. Analysis of DOX retention in (A) MCF7/Adr6c, (B) MCF7/AdrV, and (C) MCF7/AdrR cells by flow cytometry. In each graph, the solid thin line represents cells that were untreated; the solid bold line represents cells loaded with DOX for 2 h. The dotted thin and bold lines represent DOX-loaded cells after 3 or 5 h of chase. Experiments were performed twice, yielding similar results.

Table 1
Effects of chemotherapeutic drugs on Rab6c-transfected MCF7/AdrR cells[a]

Drug	MCF7/AdrV			MCF7/Adr6c-4			MCF7/Adr6c-5		
	IC50 (μM) (range, μM)	*N*	SF	IC50 (μM) (range, μM)	*N*	SF	IC50 (μM) (range, μM)	*N*	SF
DOX	0.975			0.115			0.404		
	0.9–1.0	5	1	0.11–0.14	5	8.5	0.39–0.42	5	2.4
TAX	0.885			0.215			0.975		
	0.8–0.95	5	1	0.2–0.26	5	4.1	0.9–1.1	5	0.91
VBL	0.155			0.04			0.129		
	0.13–0.17	4	1	0.03–0.05	4	3.9	0.12–0.135	4	1.21
VCR	0.51			0.14			0.49		
	0.5–0.6	5	1	0.13–0.15	5	3.7	0.48–0.495	5	1.04
CISP	0.778			0.85			0.619		
	0.76–0.82	3	1	0.74–0.95	3	0.92	0.57–0.67	3	1.26
VP-16	66.85			40.85			36.45		
	60.8–70.0	3	1	40.0–42.0	3	1.64	32.0–40.0	3	1.83

[a] *N* represents the number of times each assay was repeated. SF represents the drug sensitive factor. For DOX, the mean IC50 ± SD of the MCF7/Adr6c-4 and -5 cells was 0.115 ± 0.007 and 0.404 ± 0.016 μM, whereas that of the control cell was 0.975 ± 0.035 μM. For TAX, the mean IC50 ± SD of the MCF7/Adr6c-4 and -5 cells was 0.215 ± 0.021 and 0.975 ± 0.064 μM, whereas that of the control cell was 0.885 ± 0.05 μM. For VBL, the mean IC50 ± SD of the MCF7/Adr6c-4 and -5 cells was 0.04 ± 0.014 and 0.129 ± 0.008 μM, whereas that of the control cell was 0.155 ± 0.021 μM. For VCR, the mean IC50 ± SD of the MCF7/Adr6c-4 and -5 cells was 0.14 ± 0.009 and 0.49 ± 0.005 μM, whereas that of the control cell was 0.51 ± 0.014 μM. For CISP, the mean IC50 ± SD of the MCF7/Adr6c-4 and -5 cells was 0.85 ± 0.012 and 0.619 ± 0.009 μM, whereas that of the control cell was 0.778 ± 0.028 μM. For VP-16, the mean IC50 ± SD of the MCF7/Adr6c-4 and -5 cells was 40.85 ± 0.495 and 36.45 ± 5.16 μM, whereas that of the control cell was 66.85 ± 2.616 μM. Based on the IC50s, we determined that the Rab6c-4 cells exhibited 8.5 ($P < 0.019$), 4.1 ($P < 0.036$), 3.9 ($P < 0.024$), and 3.7 times ($P < 0.001$) more sensitivity to DOX, TAX, VBL, and VCR than the control cells. The Rab6c-5 cells exhibited 2.4 times ($P < 0.03$) more sensitivity to DOX than the control cells.

fluorescence, which clearly demonstrated the effect of *Rab6c* on DOX retention in the host cells (Fig. 5A). These results indicate that the increased sensitivity of MCF7/Adr6c to DOX could be due to prolonged drug retention by *Rab6c*.

3.6. Growth inhibition assays of cloned sublines for different drugs

We have experimentally proven that *Rab6c* is involved in DOX resistance. In an attempt to observe stronger effects, stable individual clones were generated by limiting dilution. Expressions of the exogenous *Rab6c* in two clones [MCF7/Adr6c-4 (#4) and MCF7/Adr6c-5 (#5)] were verified by RT-PCR as previously described. The IC50s for these cloned cells in six drugs, DOX, CISP, TAX, VBL, VCR, and VP-16, were evaluated. The results demonstrated that clone #4 exhibited a stronger phenotype than clone #5. The *Rab6c* transgene caused clone #4 to be approximately 8.5, 4.1, 3.9, and 3.7 times more sensitive to DOX, TAX, VBL, and VCR than the MCF7/AdrV control (Fig. 6). However, clone #4 and the control exhibited similar sensitivities to CISP and VP-16. The transgene increased the clone #5′ sensitivity approximately 2.4 times to DOX (Fig. 6), but did not increase its sensitivity to the other drugs compared with the control. The detailed results are summarized in Table 1.

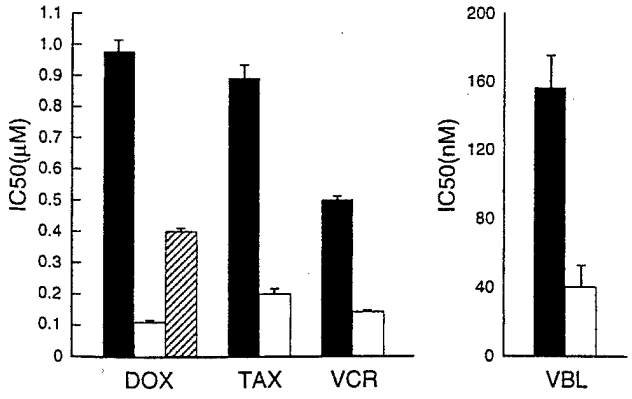

Fig. 6. The results of growth-inhibition assay for MCF7/AdrV, MCF7/Adr6c-4, and MCF7/Adr6c-5 cells. The black, white, and striped vertical columns represent the IC50 of MCF7/AdrV, MCF7/Adr6c-4, and MCF7/Adr6c-5 cells (mean of four or five experiments) to each drug (marked below). The thin bars represent SD.

4. Discussion

A new member of the *Rab* gene family, *Rab6c*, has been discovered by a DNA fragment that was hypermethylated in MCF7/AdrR cells. *Rab6c*'s MDR-related function in MCF7/AdrR cells was consequently explored.

It has been noted that, at a protein level, Rab6c closely resembles Rab6. There are only three amino acid substitutions in Rab6c at positions 62 (Ile), 87 (Ala), and 88 (Ala); this compared with Rab6, where these

positions are Val, Thr, and Val (Zahraoui et al., 1989). However, at a DNA level, owing to a 96 bp region that was found to harbor 31 bp variations in *Rab6c*, one can easily distinguish these two genes. Recently, the cDNA sequence that is identical to *Rab6c* was also obtained and directly submitted to the NIH GeneBank by two scientific groups (Barr, F.A., and Opdam, F.J.M.).

Our studies indicate that *Rab6c* is involved in drug resistance. First, we found that *Rab6c* expression was seven times lower in MCF7/AdrR cells compared with the parental MCF7/WT cells, and 3.5 times lower in MES-SA/Dx5 relative to MES-SA cells. The degree of decreased *Rab6c* transcription in MES-SA/Dx5 was obviously less than that in MCF7/AdrR cells. This result seems to correlate with the drug-resistant strength of these two cell lines. For example, based on our experience, MCF7/AdrR exhibits a more than 800-fold resistance to DOX relative to MCF7/WT cells, whereas MES-SA/Dx5 exhibits 100-fold resistance to DOX relative to its parental cell line (Harker and Sikic, 1985). However, whether *Rab6c* was also involved in the MDR phenotype in MES-SA-/Dx5 cells remains to be determined. Second, DOX uptake analysis suggests that expression of *Rab6c* in MCF7/AdrR cells prolongs the drug retention in the host cells, which could be the cause of heightened sensitivity to DOX. Third, by evaluating the IC50 values of *Rab6c* stable transfected MCF7/Adr6c populations and clones, we found that they were more sensitive and to different extents to a variety of anti-cancer drugs than the MCF7/AdrV control cells.

Our studies provide evidence that, in addition to the known drug-resistant genes MDR1, glutathione transferase and glutathione peroxidase (positive regulators), a small G protein encoded by *Rab6c* (negative regulator), also participates in developing the drug-resistant phenotype in MCF7/AdrR cells. This finding did not surprise us, considering the structural similarities of *Rab6* and *Rab6c*, and the fact that Rab6 is associated with Golgi and trans-Golgi network membranes (Martinez et al., 1994; Mayer et al., 1996), and acts as an inhibitor in anterograde transport within this organelle (Martinez et al., 1994, 1997; Martinez and Goud, 1998). This feature makes one contemplate whether *Rab6* genes inhibit anterograde transport, or stimulate retrograde transport of a drug.

Our studies suggest that multiple biological processes are involved in the development of MDR in cancer cells. The search for those unknown elements and the study of their biological functions will be of great help in understanding the mechanisms that cause clinical multidrug resistance.

Acknowledgements

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WTH3, a new member of the *Rab6* gene family, and multidrug resistance

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Abstract

The *WTH3* gene was obtained by a DNA fragment isolated by the methylation-sensitive representational difference analysis technique due to its hypermethylation in the human multidrug resistant (MDR) breast cancer cell line MCF7/AdrR. The *WTH3* gene product is 89% and 91% identical to the human Rab6 and Rab6c proteins, but possesses an elongated C-terminal region which contains 46 extra amino acids. Nevertheless, we consider the *WTH3* gene a new member of the *Rab6* gene family. Semi-quantitative reverse transcriptase–polymerase chain reaction results showed that *WTH3* was 15 and 4 times downregulated in MCF7/AdrR and MES-SA/Dx5, a human MDR uterine sarcoma cell line, as compared to their non-MDR parental cell lines. Permanent expression of the *WTH3* transgene in MDR cell lines increased to varying degrees their sensitivity to several anticancer drugs, which included doxorubicin, taxol, vinblastine, vincristine, and etoposide, as compared to the control sublines transfected with the empty vector. Flow cytometry and fluorescence microscope experiments suggest that the *WTH3* transgene stimulated the host's uptake and retention of DOX. Our results imply that the *WTH3* gene plays a role(s) in MDR phenotype development in vitro.

Keywords: Methylation-sensitive representational difference analysis; G protein; Gene transfection; Drug resistance

1. Introduction

Although *MDR1* and *MRP* genes [1–6], as well as other gene products, such as lung resistance-related protein (LRP) and glutathione transferase [7–9], have been widely recognized as factors involved in MDR development, the clinical MDR phenomenon is still not completely understood. Clearly other unknown drug resistant mechanisms are at work [10,11]. Therefore searching for those mechanisms, with the help of modern techniques, may provide information of great importance in understanding the etiology of clinical MDR.

One strategy, which could be utilized to uncover these hidden systems, is the study of DNA methylation. This event suppresses gene expression which may cause distinct cellular phenotypes [12–14]. For example, a correlation between DNA methylation, gene expression, and drug resistance has been reported by several scientific groups [15–19]. In addi-

Abbreviations: aa, amino acid; bp, base pair(s); cDNA, DNA complementary to RNA; MS-RDA, methylation-sensitive representational difference analysis; MDR, multidrug resistance; MTT, 3-[4,5-dimethylthiazol-*Z*-yl]-2-5-diphenyl-tetrazolium bromide

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tion, a methylated site is usually found close to the 5′ end of the gene to be regulated [20–22], and therefore a differentially methylated DNA fragment could be a useful tool in the search for genes related to MDR. Considering this we employed methylation-sensitive representational difference analysis (MS-RDA) [23,24], which is an RDA [25] based technology, to search for DNA fragments which were hypermethylated in a MDR human breast cancer cell line, MCF7/AdrR, but not in its non-MDR parental cell line, MCF7/WT. Both cell lines were gifts from Dr. K. Cowan of the National Cancer Institute [26].

As a result, a hypermethylated DNA fragment, *W3*, was isolated by MS-RDA. The study of *W3* has led to the discovery of two genes, *Rab6c* [27] and *WTH3*. In this report we present our findings related to *WTH3*, which was acquired by performing the 3′-RACE (rapid amplification of cDNA ends) technique based on the *W3* sequence. *WTH3* is homologous to the human *Rab6* and *Rab6c*/*Rab6A′* genes [27–29] but possesses its own unique feature, an extended 46-amino-acid C-terminal region (recently, this gene has been documented by another group [30]). Consistent with its hypermethylated status, the *WTH3* gene was 15 times less expressed in MCF7/AdrR than in MCF7/WT cells. In addition, it was four times less expressed in the MES-SA/Dx5 cell line (its MDR phenotype was induced from the parental cell line, MES-SA, by doxorubicin (DOX) (ATCC Inc.) [31,32]) as compared to MES-SA. Research on the stable MCF7/AdrR and MES-SA/Dx5 cell lines which contained the *WTH3* transgene suggest that *WTH3* reversed the host cells' MDR phenotype to several anticancer drugs tested and increased DOX retention of the host cells. Our studies indicate that the *WTH3* gene, functioning as a negative regulator, was involved in the evolution of drug resistance in the working model systems. Whether *WTH3* is also involved in clinical drug resistance is currently under investigation.

2. Experimental procedures

2.1. Human cell lines and culture conditions

MCF7/AdrR, MCF7/WT, MES-SA/Dx5, and MES-SA cells were grown under the conditions as described [27].

2.2. MS-RDA

To study hypermethylation events in MCF7/AdrR cells, MCF7/WT DNA was used as tester, while MCF7/AdrR DNA was used as driver. MS-RDA was performed as described [23].

2.3. Amplicon and genomic methylation sensitive Southern analysis

Amplicon Southern blot was performed as described [23]. Two hundred and fifty ng of tester and driver amplicon DNA were utilized. Genomic methylation sensitive Southern blot was performed as described [23].

2.4. DNA Sequencing

Polymerase chain reaction (PCR) products were sequenced using a Dye-Labeled Sequence kit (Perkin Elmer) under conditions specified by the manufacturer.

2.5. Obtaining the WTH3 Gene by 3′-RACE

A unique sequence, 5′-GATGGAACAATCGGGCTTCG-3′ (PW3-1), which is located in the middle of the *W3* fragment, was designed as a PCR primer to obtain the 3′ end portion of the *WTH3* gene from a combined human cDNA library (Quick-Screen Human cDNA library Panel, Clontech). The antisense primer (PV-1), 5′-ACGACTCACTATAGGGCGAATTGGC-3′, was designed based on the vector sequence. PCR amplification was performed using the Advantage GC KlenTaqPolymerase Mix kit (Clontech) following the manufacturer's instructions. A 1.3 kb PCR product was generated and sequenced. Based on the sequencing information, another *W3*-specific primer, 5′-AAACAGTCAGCGAAGGGGGT-3′ (PW3-RACE1), which is close to the stop codon, was designed to perform RACE to obtain another PCR product to verify that the original PCR product is our true target. To acquire an intact gene, PW3-1 was paired with another primer, 5′-CCCAAGCTTGACTTTTTTTGTGCTTGTCAAGC-

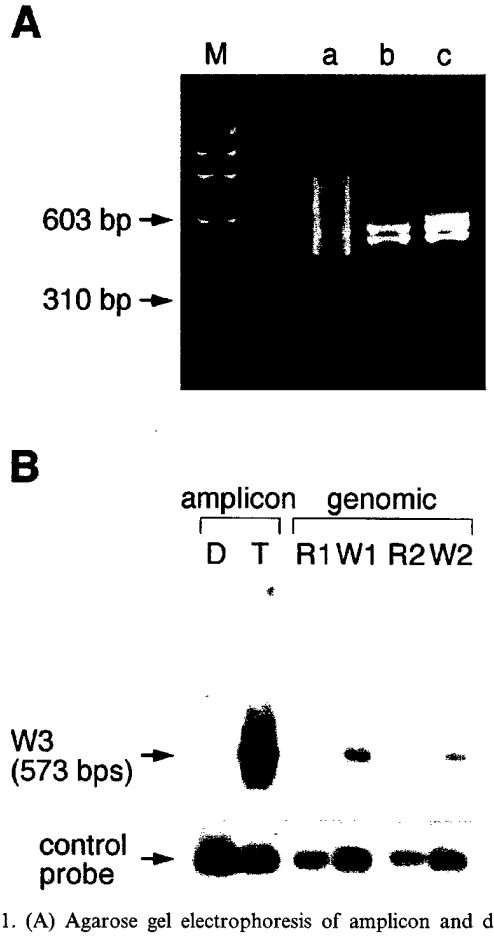

Fig. 1. (A) Agarose gel electrophoresis of amplicon and difference products generated by MS-RDA from MCF7/WT DNA. Lane M represents *Hae*III ϕ174 markers. Lane a represents the tester amplicon. Lanes b and c contain the difference products after first and second round subtraction hybridization/PCR amplification. (B) Results of the amplicon and genomic Southern blot for hypermethylated *W3*. The arrows indicate the fragments that are hybridized with the radiation labeled *W3* and control probes. In lanes D and T, each probe hybridizes with 250 ng of driver (MCF7/AdrR) and tester (MCF7/WT) amplicon DNA. In lanes R1 and W1, the *W3* probe hybridized with 6 μg of *Hpa*II-digested genomic DNA isolated from MCF7/AdrR and MCF7/WT cells, respectively. In Lanes R2 and W2, each probe hybridized with 6 μg of *Msp*I-digested genomic DNA also isolated from MCF7/AdrR and MCF7/WT. The completion of enzymatic digestion for all genomic DNAs was confirmed by a control probe which was a background DNA fragment isolated by MS-RDA.

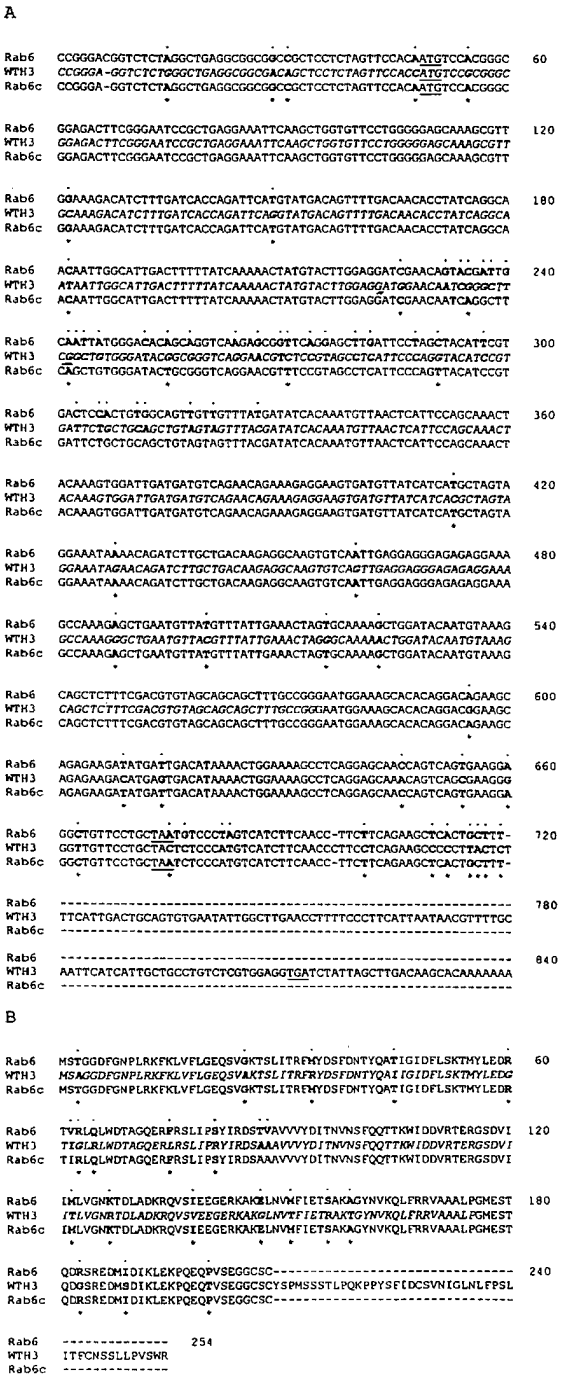

Fig. 2. The comparison of nucleotide sequences between *WTH3*, *Rab6*, and *Rab6c* (A) and the comparison of amino acid sequences between WTH3, Rab6, and Rab6c (B). The *W3* fragment isolated by MS-RDA is in italics. The nucleotide and amino acid substitutions between the three genes are in bold type. The differences between *WTH3* and *Rab6* and their products are marked with dots, while the differences between *WTH3* and *Rab6c* and their products are marked with stars. The start and stop codons of the genes are underlined.
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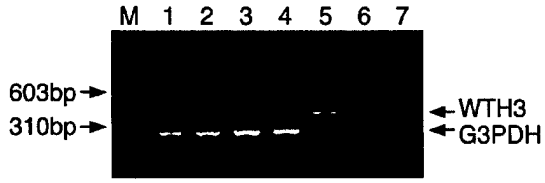

Fig. 3. Results of *WTH3*-specific SQRT–PCR. M represents *Hae*III ϕ174 markers. The cell lines used to prepare cDNA for simultaneously generated *WTH3* and *G3PDH* PCR products are indicated by numbers: 1, MES-SA/Dx5; 2, MES-SA; 3, MCF7/AdrR; 4, MCF7/WT. Lanes 5 and 6 contain *WTH3* and *G3PDH* PCR products generated from cDNA prepared from MCF7/WT cells, which served as positive controls. Lane 7 is the PCR negative control.

3′ (PW3-RACE2), which is located beyond the stop codon and followed by an artificial *Hin*dIII restriction enzyme site (for cloning purposes), to generate the 3′ end of the gene. This PCR product was digested by *Bgl*II, an endogenous site, and *Hin*dIII. To obtain the intact *WTH3* gene, the *Bgl*II/*Hin*dIII fragment was then subcloned into the pUC118-W3 plasmid containing the *W3* sequence, which was also digested by *Bgl*II, the corresponding endogenous site, and *Hin*dIII, which is in the polylinker, to release the truncated 3′ end region.

2.6. Semi-quantitative reverse transcriptase–polymerase chain reaction (SQRT–PCR) and RT–PCR

Total RNAs were isolated from the cell lines by the RNA isolation kit, RNA STAT-60 (TEL-TEST). The first-strand cDNA was synthesized by Super-Script Preamplification System Kit (Life Technologies). SQRT–PCR was performed by utilizing PW3-1, the *WTH3* gene sense primer, and the antisense primer, 5′-GCTGCTACACGTCGAAAGAGC-3′, while the cDNAs of MCF7/AdrR, MCF7/WT, MES-SA/Dx5, and MES-SA served as templates. The length of the *WTH3* PCR product was 341 bp. The sense and antisense primers for *G3PDH* (internal control) were 5′-CGGAGTCAACGGATTTGGT-CGTAT-3′ and 5′-AGCCTTCTCCATGGTGGT-GAAGAC-3′. The length of the *G3PDH* PCR product was 284 bp. To confirm exogenous *WTH3* gene expression in the stable cell line, PW3-RACE1 was employed as the sense primer. A sequence in the polylinker of the pcDNA3.1 (Invitrogen) vector, 5′-CACTGTGCTGGATATCTGCAG-3′ (PV-2), was

Table 1
Effects of drugs on *WTH3* transfected MES-SA/Dx5 clone cells

Drugs	ME-V			ME-2			ME-4			ME-7		
	IC_{50} (nM) (range, nM)	*N*	SF	IC_{50} (nM) (range, nM)	*N*	SF (range, nM)	IC_{50} (nM)	*N*	SF	IC_{50} (nM) (range, nM)	*N*	SF
DOX	770			22			93.3			54		
	(700–900)	5	1	(9–26)	5	35	(85–130)	5	8.3	(45–100)	5	14.3
TAX	615			2.85			28			20.5		
	(490–700)	4	1	(1.92–3.0)	4	216	(16–30)	3	22	(9–25)	3	30
VBL	59			1.3			7			3.6		
	(45–70)	4	1	(0.3–1.5)	7	45	(5–13)	4	8.4	(2.8–5.8)	4	16.4
VCR	408			2.35			30.7			14.5		
	(380–480)	5	1	(0.2–3.2)	11	174	(28–60)	5	13	(6–35)	5	28
VP-16	8900			340			2300			1050		
	(6900–11000)	4	1	(320–500)	4	26	(500–2700)	4	4	(600–1900)	4	8.5
CISP	1400			810			1413			1180		
	(1200–1500)	4	1	(640–840)	4	1.7	(1325–1775)	4	1	(420–1400)	4	1.2

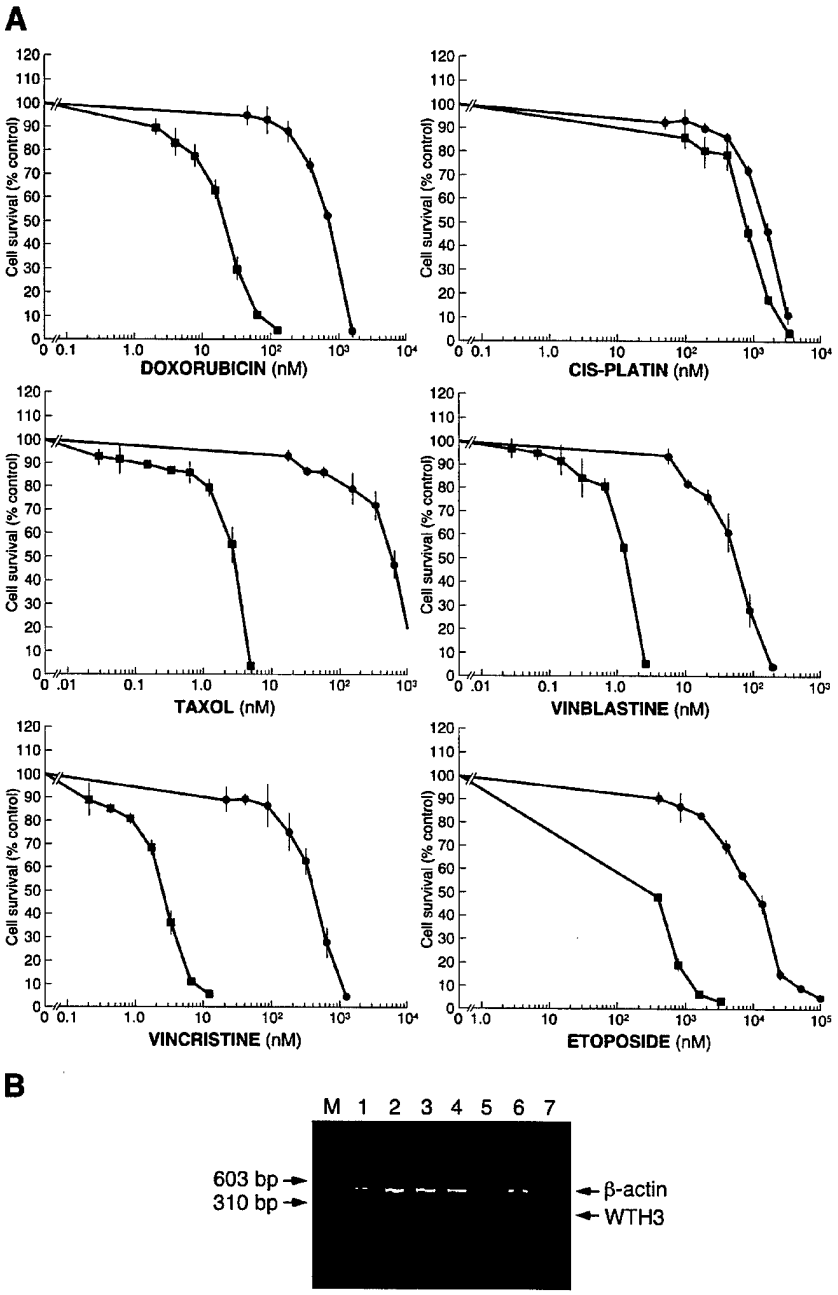

Fig. 4. Growth inhibition of ME-2 cells caused by different concentrations of DOX, CISP, TAX, VBL, VCR, and VP-16 (A) and QRT–PCR of the expressed exogenous *WTH3* gene in ME-2, ME-4, and ME-7 cells (B). (A) The line formed by linking the solid circles or squares represents the survival rate of ME-V and ME-2 cells (mean of more than four experiments), respectively. The thin bars represent standard deviation. (B) Lane M represents *Hae*III ϕ174 markers. Lanes 5 and 6 contain the positive controls for *WTH3* and *β-actin*. The product in lane 5 was generated by using cDNA prepared from ME-2 RNA and *WTH3*-specific primers. The product in lane 6 was generated from cDNA prepared from MES-SA/Dx5 cells. Lanes 1, 2, and 3 contain simultaneously generated *WTH3* and *β-actin* PCR products by utilizing cDNAs which were prepared from ME-2, ME-4, and ME-7 cells. Lane 4 only contains *β-actin* generated from cDNA prepared from ME-V cells, although *β-actin*- and *WTH3*-specific primers were simultaneously added. Lane 7 is the negative control.

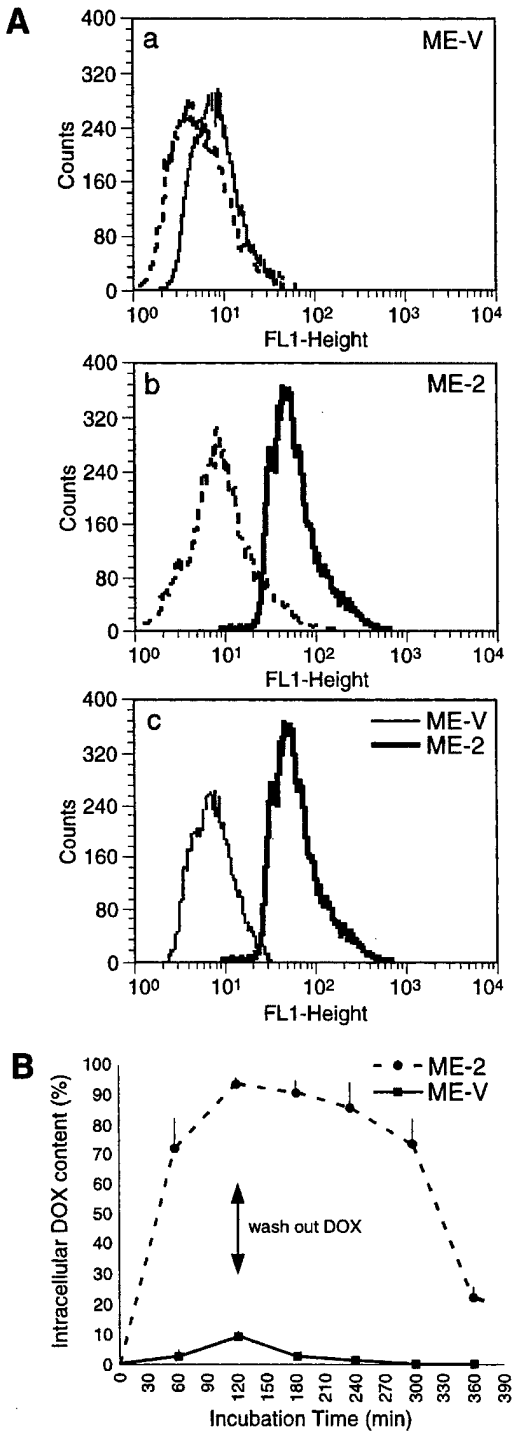

Fig. 5. Analysis of DOX uptake in ME-V and ME-2 cells by flow cytometry. DOX uptake in each cell group is presented by a peaked graph (A), and DOX uptake and retention in each group is presented by a curved graph (B). (A) Comparison of intercellular fluorescence in ME-V versus ME-2 cells. a, ME-V, at 0 (dashed line) and 2 h incubation with DOX (thin solid line); b, ME-2, at 0 (dashed line) and 2 h incubation with DOX (thick solid line); c, the comparison of accumulated DOX fluorescence in ME-V (thin solid line) and ME-2 (thick solid line) at 2 h incubation. (B) Cells were treated with DOX for 2 h and then washed. The cells remained in a DOX-free medium for up to 4 h. The intercellular fluorescence at different time points was measured by a flow cytometer. The solid and dashed lines represent ME-V and ME-2 cells, respectively. The solid points are the means of duplicated determinations. The vertical bars represent the upper or lower range at that data point.
←

used as antisense primer to synthesize the *WTH3* transgene fragment (226 bp). The sense and antisense primers for generating the *β-actin* fragment (495 bp) which served as a control were 5′-GACGACATG-GAAGATCTGG-3′ and 5′-ATCGGGCAGCTCG-TAGCTCTTCC-3′. The PCR and quantification of PCR products were performed as described [27].

2.7. Generation of stable cell lines

The PCR-generated *WTH3* gene was subcloned into the mammalian expression vector, pcDNA3.1, to create pcDNA3.1/WTH3. The gene sequence was confirmed by DNA sequencing. MCF7/AdrR and MES-SA/Dx5 cells grown to 60% confluence in 60-mm dishes were transfected with 2 μg of pcDNA3.1/WTH3, or pcDNA3.1 (control) plasmid using a calcium phosphate precipitation kit (5 Prime 3 Prime). The transfected MCF7/AdrR and MES-SA/Dx5 cells were maintained in medium supplied with 200 and 250 μg/ml of neomycin analogue G418 (Gibco), respectively. Stable G418 resistant populations were acquired after 2 weeks of selection. The individual clones were obtained by limiting dilution.

2.8. Cell growth inhibition

Approximately 1×10^3/well of *WTH3* or the empty vector transfectants were seeded in a 96-well plate (Corning Costar) and grown overnight. DOX, cisplatin (CISP), taxol (TAX), vinblastine (VBL), vincristine (VCR), and etoposide (VP-16) (Sigma) were se-

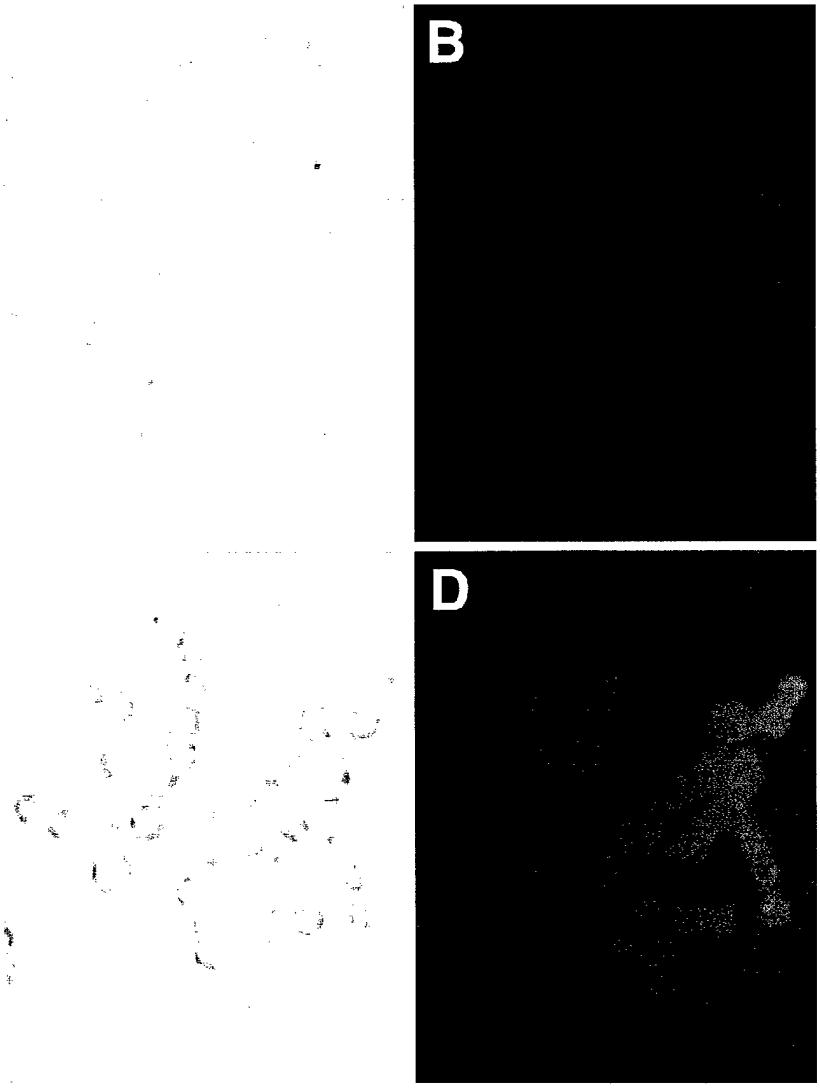

Fig. 6. Fluorescence microscopy of DOX accumulation and distribution assay. ME-V (A,B) and ME-2 (C,D) cells were loaded with DOX for 2 h (A,C, bright field; B,D, fluorescent field). Experiments were performed three times giving similar results. ×150.

rially diluted into ten concentrations. Every group of four wells received one drug concentration. After a 6-day incubation period, the cells were treated with 3-[4,5-dimethylthiazol-*Z*-yl]-2-5-diphenyl-tetrazolium bromide (MTT) which stains living cells. IC_{50} was quantitatively evaluated as described [27].

2.9. Flow cytometry

Approximately 5×10^5/well of *WTH3* or the empty vector transfected cells were seeded in six-well dishes. Since the intracellular fluorescence intensity was tested at seven time points, seven wells for each transfectant group were prepared. One well for each group was not treated by DOX and served as the 0 time control. Each group of six wells was incubated with 30 μg/ml DOX in 1 ml of culture media for 2 h at 37°C in 5% CO_2. To measure the intracellular fluorescence intensity, the cells were washed twice in ice cold PBS and trypsinized. Cells were centrifuged at 1000 rpm for 5 min to pellet and then resuspended in 300 μl of 1% paraformaldehyde.

The intracellular DOX fluorescence content was analyzed by flow cytometry as described [33]. The fluorescence intensity was measured at 1- and 2-h intervals during the DOX incubation period. The remaining four wells of each transfectant were then washed twice with ice-cold PBS and incubated in a DOX-free culture medium for 4 h. The intensity of fluorescence at 1, 2, 3, and 4 h of chase was measured.

2.10. Fluorescence microscopic assay

Approximately 5×10^4/well of *WTH3* or the empty vector transfectants were cultured in six-well dishes. Individual wells were incubated with 10 μg/ml DOX in medium for 2 h. Medium was then removed and the cells were washed with ice-cold PBS, followed by the addition of fresh media without DOX. Cells were immediately examined for fluorescence at 488 nm excitation wavelength by fluorescence microscopy.

The GenBank accession number for *WTH3* is AF309646 (it will be released when the gene is published), and that for *W3* is AF11936.

3. Results

3.1. Isolation of a hypermethylated DNA fragments by MS-RDA

After two rounds of DNA subtractive hybridization and PCR amplification, individual fragments, considered difference products, were isolated from the MCF7/WT cells (Fig. 1A). These fragments were then subcloned into the pUC118 vector, and the candidate inserts were screened by amplicon Southern analysis. One of the candidate probes was *W3*, since it only hybridized with the tester, but not driver amplicon DNA (Fig. 1B). To prove that *W3* was hypermethylated in MCF7/AdrR cells, it was used as the probe to perform methylation sensitive Southern blot. Both cell lines' genomic DNAs were digested by *Hpa*II or the *Msp*I enzyme. *Msp*I is an isoschizomer of *Hpa*II, but not sensitive to C^mCGG. Therefore, *Msp*I could cleave a methylated site which could not be cut by *Hpa*II. Southern analysis results indicated that *W3* was hypermethylated in the MCF7/AdrR DNA because *Msp*I digestion released a band which was not cleaved by the *Hpa*II enzyme (Fig. 1B).

The *W3* fragment was sequenced and found to contain 573 bp which shared 89% and 96% identity with the corresponding portion of the human *Rab6* and *Rab6c*/*Rab6A'* genes [27–29]. Based on protein sequence analysis, *W3* encodes 175 amino acids without an interruption. In addition, at its 5′ end there is a stop codon, TAG, that is 27 bp away from a start codon. This suggested that the complete N terminus of the putative gene was included in the *W3* fragment, however, the C-terminal region was missing (Fig. 2A,B).

3.2. Obtaining the WTH3 gene by 3′-RACE

To acquire the putative full length cDNA of *W3*, the RACE technique was employed where a combined cDNA panel of different cell types (a commercial product of Clontech) was used as a template. To obtain the missing 3′ end portion of the candidate gene, a primer, PW3-1, paired with an antisense primer, PV-1, which was the vector's sequence close to the junction of the cDNA insertion, were utilized to perform PCR amplification. PW3-1 is a specific primer for *W3*, since in its 20 bp length sequence there are eight and three substitutions as compared to the corresponding region in *Rab6* and *Rab6c*. This specific feature was confirmed by the fact that no PCR product was generated from the plasmid containing *Rab6* or *Rab6c* sequences when PW3-1 and an antisense primer, a common sequence in *Rab6*, *Rab6c*, and *W3*, were used (data not shown). As a result of PCR amplification, a single 1.3 kb length PCR fragment was generated. This fragment was then electrophoresis-purified and sequenced. Our findings suggested that the PCR product was our target since the initial 349 bp sequence matched perfectly to the corresponding region of the *W3* sequence. Furthermore, in the middle of the PCR product, there was a stop codon which ended a 220-amino-acid stretch. To verify these results, another primer, PW3-RACE1, which is beyond *W3*'s 3′ region and before the stop codon, was used to perform a second RACE. PW3-RACE1 contains 4 bp substitutions as compared to the corresponding regions in *Rab6* and *Rab6c*, and is specific for the extended *W3* sequence. A PCR product approxi-

mately 800 bp in length was amplified. Sequence analysis confirmed that the N-terminal was identical to the corresponding sequence of the first PCR product. To further prove the accuracy of the PCR products, we generated the missing 3′ end portion of the target gene, *W3-3P*, from two other human cDNA libraries (Kidney and Brain cDNA libraries, Clontech). This was accomplished by utilizing the PW3-1 primer paired with the PW3-RACE2 primer which was located beyond the stop codon. Sequence analysis confirmed that both PCR products were identical to the two sequences previously generated by RACE. Therefore, we were convinced that the entire sequence information for the target gene had been obtained. We named this gene *WTH3* due to the fact that its truncated portion was originally isolated from MCF7/*WT* by *H*pa II cleavage MS-RDA.

To acquire an intact *WTH3* gene, the *W3-3P* fragment was digested with *Bgl*II and *Hin*dIII. The *Bgl*II site is an endogenous sequence, while the *Hin*dIII site was added into the PW3-RACE2 primer for cloning purposes. At the same time, the pUC118-W3 construction, which contains the *W3* DNA fragment isolated by MS-RDA, was also digested with *Bgl*II and *Hin*dIII to release the truncated region and create pUC118-W3$^-$. The enzymatic treated *W3-3P* was then ligated to the linearized pUC118-W3$^-$ to obtain the full-length *WTH3* gene. Differing from *Rab6* and *Rab6c*, which both encode a 208-amino-acid product, the *WTH3* cDNA encodes a 254-amino-acid protein. In the corresponding 718 bp sequences, *WTH3* is 92% and 95% identical to *Rab6* and *Rab6c* (Fig. 2A), while in the corresponding 208-amino-acid sequences, WTH3 is 89% and 91% identical to Rab6 and Rab6c (Fig. 2B). In addition, compared to the *Rab6* and *Rab6c* genes, *WTH3* has an elongated C-terminal region consisting of 46 amino acids (Fig. 2B). Recently, the Wiemann group reported that they have obtained 500 new cDNAs, among these, a *Rab6-like* gene was 100% identical to *WTH3* [30]. This finding verifies the sequence accuracy of the *WTH3* gene.

3.3. The WTH3 gene was underexpressed in MCF7/AdrR and MES-SA/Dx5 cells

Since the N-terminal portion of the *WTH3* gene, *W3*, was hypermethylated in MCF7/AdrR cells, the *WTH3* gene's expression level in MCF7/AdrR and MCF7/WT cells was analyzed by SQRT–PCR using gene-specific primers. The predicted size of the *WTH3* PCR product was 341 bp, while 284 bp was the anticipated length of the *G3PDH* gene which served as a quantitative control. The results showed that the expression of *WTH3* in MCF7/AdrR cells was 15 times less than that in MCF7/WT cells (Fig. 3). In addition, the *WTH3* gene's expression level was evaluated in the MDR cell line MES-SA/Dx5 and its non-MDR counterpart MES-SA. We found that *WTH3* was four times less expressed in MES-SA/Dx5 as compared to MES-SA cells (Fig. 3).

3.4. Drug-induced WTH3 transfectants growth inhibition assays

Hypermethylation and low expression of the *WTH3* gene in MCF7/AdrR and MES-SA/Dx5 cells indicated that this gene might be a negative regulator for drug resistance. To test this hypothesis and obtain reliable results, both MCF7/AdrR and MES-SA/Dx5 were transfected with the *WTH3* gene to generate stable cell lines. Another reason for using the MES-SA/Dx5 line was that it exhibited a much weaker MDR phenotype than MCF7/AdrR. Therefore, the *WTH3* gene could have a stronger influence on MES-SA/Dx5 than MCF7/AdrR cells.

The *WTH3* gene was generated by PCR and subcloned into the pcDNA3.1 vector to create pcDNA3.1/WTH3. This construction and the vector were separately introduced into the host cells by calcium phosphate precipitation procedure. The transfected cells were maintained in medium containing G418 for selecting stable transformed populations. After verifying, by measuring their IC_{50}s, that the stable populations harboring the transgene exhibited higher sensitivity to DOX than the controls integrated with the empty vector, limiting dilution was carried out to obtain stable cell clones. Three MCF7/AdrR and five MES-SA/Dx5 individual transfectants were selected. Limiting dilution procedures were also carried out to obtain five individual MCF7/AdrR or MES-SA/Dx5 transfectants integrated with the empty vectors (negative controls). The expression of the exogenous *WTH3* gene in each clone was con-

firmed by RT–PCR, where the gene-specific primer PW3-1 and pcDNA3.1 poly-linker primer PV-2 were used. All clones, along with their controls, were utilized for drug-induced cell growth inhibition assays. IC_{50}s for the anticancer drugs DOX, CISP, TAX, VBL, VCR, and VP-16 were evaluated. We found that the transgene increased the MCF7/AdrR clones' sensitivity to DOX, TAX, VBL, and VCR by factors ranging from 2- to 6-fold (data not shown). However, the transgene had a much stronger influence on four out of five MES-SA/Dx5 clones. For example, both clones #2 (ME-2) and #8 (ME-8) had significant increased sensitivity to DOX, TAX, VBL, VCR, and VP-16 as compared to the five controls containing the empty vector (ME-V). The sensitivity of clones #4 (ME-4) and #7 (ME-7) to the same drugs was also elevated by the transgene but to a lesser extent. Clone #5, which expressed a very low level of the transgene, exhibited a drug resistant phenotype similar to that of all five control sublines (data not shown). In the next paragraph, detailed information on the IC_{50} measurements of clones ME-2, ME-4, and ME-7 is presented.

To determine the IC_{50}s, ME-2, ME-4, ME-7, and five ME-V sublines were kept in media without the drug, or with the other ten drug concentrations for 6 days. More than three individual experiments were performed for each cloned cell line. The IC_{50} measurements found that the *WTH3* transgene increased the host clone's sensitivity to DOX, TAX, VBL, VCR, and VP-16, but not to CISP, to varying degrees as compared to the control cell lines which exhibited the original MDR phenotype (Table 1). Since all five ME-V controls had similar resistance to the drugs tested, only the results obtained from one ME-V were listed. The sensitivity of the ME-2 clone to DOX, TAX, VBL, VCR, and VP-16 was 35, 216, 45, 174, and 26 times greater than that of the control cells (Table 1 and Fig. 4A). The ME-4 clone's sensitivity to the same anticancer drugs was 8, 22, 8, 13, and 4 times higher than the control cells, while that of the ME-7 clone was elevated 14, 30, 16, 28, and 8.5 times relative to the control (Table 1). Expression of the *WTH3* transgene in ME-2, ME-4, and ME-7 was verified by RT–PCR (Fig. 4B). Densitometer analysis found that the expression level of the transgene in ME-2 was 1.6 and 2.2 times higher than that in ME-7 and ME-4, which could be a reason for ME-2 exhibiting higher drug sensitivity.

3.5. *Flow cytometry assay for DOX uptake and retention in ME-2 cells*

DOX is fluorescent, and this attribute provides easy monitoring of its intracellular accumulation by flow cytometry. Thus, ME-V and ME-2 cells were incubated with DOX for 2 h, after which the cells were washed and remained in a medium without DOX for 4 h. DOX uptake and retention in the cells was quantitatively determined at different time points (see Section 2). The ME-V cells displayed no significant increase in cellular fluorescence after DOX incubation. The ME-2 cells, which contained the *WTH3* transgene, however, displayed greatly increased DOX uptake (Fig. 5A). Furthermore, the intensity of fluorescence in ME-2 and ME-V cells was measured at four time points after the cells were washed with PBS buffer. The results showed that the fluorescence remained much stronger even after 4 h of chase in ME-2 than that in ME-V cells (Fig. 5B). These findings clearly demonstrate the positive effect of the *WTH3* gene on DOX uptake and retention in the host cells.

3.6. *Fluorescence microscopy of DOX accumulation in ME-2 cells*

Flow cytometry experiments found that the *WTH3* gene stimulated the host cells' uptake and retention of DOX. To visually determine the intercellular location of the accumulated DOX, fluorescence microscopy was performed. The ME-V and ME-2 cells were treated with DOX for 2 h, after which the drug was washed away. The fluorescence accumulation and distribution in the control and test cells were examined under a microscope (Fig. 6A–D). It was discovered that the control cells with the drug resistant phenotype only contained trace amounts of fluorescence (Fig. 6B), while strong fluorescence was displayed in the nucleus and cytoplasm of the ME-2 cells (Fig. 6D). The intensity of fluorescence continued to remain strong even after 3 h of chase in the ME-2 cells (data not shown).

4. Discussion

The *WTH3* gene was discovered due to hypermethylation of its N-terminal portion in MCF7/AdrR cells. Function analysis suggests that this gene could be involved in cellular MDR phenotype development.

WTH3 is homologous to the human genes, *Rab6* and *Rab6c*. *Rab6* is thought to control intra-Golgi transport by acting as an inhibitor [34]. Our previous studies suggest that *Rab6c* is involved in developing the MDR phenotype in MCF7/AdrR cells [27]. *Rab6c* was also named *Rab6A'* in a recent publication where it has been suggested that it is an isoform of *Rab6* due to alternative splicing. However, *Rab6A'* is not able to stimulate Golgi-to-endoplasmic reticulum retrograde transport as described previously for *Rab6* [29]. Both *Rab6* and *Rab6c* encode 208 amino acids with only three substitutions between them. *WTH3* encodes 254 amino acids with 22 and 19 substitutions as compared to Rab6 and Rab6c. These substitutions are evenly distributed, and this would indicate that *WTH3* is not an alternative spliced product of *Rab6*. Four domains of the K-ras protein involved in GTP/GDP binding are also included in WTH3 (corresponding to residues 20–27, 67–73, 123–130, and 153–159) [28]. This implies that *WTH3* encodes a small G protein. However, differing from Rab6 and Rab6c, WTH3 does not possess any cysteine near its COOH terminus. The existence of cysteine is assumed to be necessary for many G proteins' fatty acylation, membrane association, and biological function [35]. The C-terminal diversification suggests that the *WTH3* protein might have its own unique utilities, even though *WTH3* and *Rab6c* do share some biological functions.

Our studies demonstrated that the N-terminal portion of *WTH3* was hypermethylated in MCF7/AdrR and the gene was less expressed in MCF7/AdrR and MES-SA/Dx5 cells than in their parental cell lines. In addition, based on information provided by the Wiemann group, the 398 bp length region located at the N-terminus of *WTH3* was not only GC-rich (~71% of GC content) but also contained CpG islands [30]. Although the promoter of *WTH3* has not yet become available, its expression could be regulated by DNA methylation. This is because hypermethylation in promoters is often accompanied by hypermethylation in the 5′ transcribed region, and CpG islands usually extend from promoters into the 5′ transcribed region [20,36–38]. It is expected that when the promoter of *WTH3* is found, a reverse correlation between DNA methylation and gene expression will be ascertained.

To test whether increasing the *WTH3* gene transcript in MCF7/AdrR and MES-SA/Dx5 cells could convert the MDR phenotype, stable cloned cell lines were established. Several clones from each cell line were generated and used to perform drug-induced cell death experiments. The results demonstrated that the gene influenced both MDR cell lines although the effect is greater on MES-SA/Dx5 than MCF-7/AdrR. The mechanism for this disparity remains unclear. In the past, we reported that the *Rab6c* gene was involved in MDR in MCF7/AdrR cells. To date, our results suggest that another *Rab6*-like gene, *WTH3*, was also related to the MDR phenotype in two human cell lines. Differing from known MDR genes, which function as positive regulators for MDR development, the *Rab6* genes play negative regulation roles. It will be interesting to understand the biological pathway(s) of each member in the *Rab6* gene family and explore the possible relationships between them and P-glycoprotein. It has been reported that the mammalian *Rab6* gene product was located in the medial and *trans* Golgi cisternae as well as the *trans* Golgi network [29,34,39], while P-glycoprotein was localized in the plasma membrane of drug-resistant cells. However, whether there is direct or indirect interaction between Rab6 and P-glycoprotein remains unclear.

The question of whether the WTH3 protein directly interacts with P-glycoprotein also remains to be determined. This is because, differing from Rab6, WTH3 does not possess any cysteine near its carboxyl-terminal region, suggesting it might have a different cellular localization. The cellular location of WTH3 could conceivably be determined by tagging it with certain markers, such as green fluorescence protein (GFP), or, most ideally, by an antibody which could only recognize WTH3, but not Rab6 and Rab6c. When such an antibody is available, it will assist the study of determining the possible relationship between WTH3 and P-glycoprotein.

Based on our data, it is necessary to further investigate the *Rab6* genes' (*Rab6*, *Rab6c*, and *WTH3*)

involvement in MDR evolution. The results of this study could have a great impact in understanding clinical MDR development.

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